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Characterization of Group A Streptococcal R-28 Antigen Purified by Hydroxyapatite Column Chromatography

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Purified R-28 antigen from an M-protein-poor, R-antigen-rich strain of group A *Streptococcus* was prepared by sequential treatment of an acid extract of whole cells with ammonium sulfate fractionation and hydroxylapatite (HA) column chromatography. Purified R-28 antigen was eluted only with 0.10 M sodium phosphate, pH 6.7. Findings on quantitative amino acid composition, polyacrylamide gel electrophoresis pattern, and HA column elution pattern were similar but not identical to those previously reported for streptococcal M-proteins. Rabbits immunized with either HA-purified R-28 antigen or heat-killed cells developed two pepsin-sensitive, trypsin-resistant immunodiffusion lines of identity against HA-purified R-28 antigen but failed to form bactericidal antibody. One of these two lines formed a line of identity with R-28 antigen prepared by trypsinization of whole cells. The other line remained undefined, although it appeared not to be either streptococcal group A carbohydrate, M-protein, T-antigen, polyglycerophosphate, E4 antigen, or M-associated protein; by enzymatic criteria it is an R-antigen. Polyacrylamide gel electrophoresis of HA-purified R-28 antigen revealed multiple serologically active charge and size isomers. These findings suggest possible structural similarities between group A streptococcal M-proteins and R-antigens and also indicate that the same purification techniques may be utilized to study these protein antigens if the proper strain of *Streptococcus* is chosen.

Streptococcal R-antigens are non-type-specific surface proteins that have no currently identified biological function. Initially characterized in group A type 28 streptococci (14), R-antigens have subsequently been found in group A types 2, 3, 33, 43, and 48 (11, 27) and in groups B, C, G, and L (14, 17, 27). R-antigens of different serological specificities have been described (12, 14, 26, 27). Serologically specific R-antigens have shown multiple immunodiffusion precipitin lines (27) and nonhomogeneity on electrophoresis (14). R-antigens have caused confusing cross-precipitin reactions with grouping and typing antisera that contained antibodies to R-antigen (26, 27).

R-antigen has been mistaken for streptococcal type-specific M-protein (11, 14, 17), but differs significantly in that R-antigen is not type specific and is not associated with virulence and R-antibody is not related to protection against re-infection (14, 27).

Few methods have been described for purification of R-antigen (14), although many procedures have been developed for purification of M-proteins (3). In the study reported here, purified R-28 antigen from an M-protein-poor, R-antigen-rich type 28 group A *Streptococcus* was characterized. The antigen was prepared by purification methods previously described for the isolation of group A, type 12 M-protein (9, 24).(This work was presented in part at the 74th Annual Meeting of the American Society for Microbiology, Chicago, 1974.)

MATERIALS AND METHODS

Organism. Strain 0050 (M-28) R-28, a Center for Disease Control (CDC) group A laboratory strain which is poor in M-protein and rich in R-28 antigen, was originally obtained from Rebecca Lancefield (strain T28/51/1). It was stored at −20 C in Todd-Hewitt broth enriched with 5% defibrinated rabbit blood and was kindly supplied by William K. Harrell at the CDC, Atlanta. Strain 0050 (M-28) R-28 did not grow in the indirect bactericidal test unless an exceedingly large inoculum was used and initial survivors were selected for subculture and retesting, which indicates that it is relatively M-protein deficient. It lacked T-28 antigen (see Fig. 8) that was extractable by the methods of Pakula (22), McLean (16) and Erwa (2).

The organism was subcultured overnight into 10 ml of Todd-Hewitt broth and then overnight in 50 ml of Todd-Hewitt broth. The second subculture was plated onto 5% sheep blood agar plates to ensure its purity. Overnight growth of 50-ml subcultures was inoculated into a 120-liter batch of Todd-Hewitt

broth, grown overnight at 37 C with intermittent shaking, and harvested by centrifugation in a CEPA Schnell GLE continuous-flow centrifuge at 50,000 × *g*.

Purified R-28 antigen. Crude acid extracts (CAEs) and ammonium sulfate fractions were made from sedimented organisms as previously described (9) except that ammonium sulfate fractions Aa, Ab, Ba, and Bb were combined into single-volume samples. The final samples were dialyzed overnight against 0.01 M sodium phosphate, pH 6.7, dispensed into 5.0-ml samples for hydroxylapatite (HA) column chromatography, and frozen at −20 C.

HA column chromatography with stepwise elution with 0.01, 0.10, and 0.30 M sodium phosphate, pH 6.7, was performed as described previously (9), except that all sodium phosphate buffers contained 0.02% sodium azide. All protein-containing (as measured by optical density readings at 280 nm) tubes from a given buffer concentration were combined into single-volume samples. The combined fractions were analyzed for protein by the method of Lowry et al. (15) with an albumin standard and dialyzed overnight at 4 C in 0.01 M ammonium carbonate, pH 8.3, in ratios of 1 ml of sodium phosphate buffer eluate/100 ml of ammonium carbonate. Subsequently, another similar overnight dialysis was performed to achieve an overall 1:10,000 dilution of phosphate buffer in the HA fractions. HA fractions in ammonium carbonate buffer were frozen at −20 C, lyophilized to dryness, and stored at −20 C. Subsequent analyses of R-28 antigen were performed with this lyophilized material, unless otherwise noted.

PAGE. The initial polyacrylamide gel electrophoresis (PAGE) procedure was as described by Davis (1). All runs were performed at room temperature with a Buchler D.C. power supply and Hoefer electrophoresis and destaining apparatus. Selected samples were later electrophoresed in 3, 6, 9, and 12% acrylamide gels according to the method of Hedrick and Smith (7) for separation of size and charge isomers.

Gels were stained with 0.5% amido black in 7% acetic acid. Selected 300 to 500 μg protein samples were scanned in a densitometer with an integrator attachment and were photographed.

Amino acid composition. Quantitative determinations were performed by Analytical BioChemistry Laboratories, Inc., Columbia, Mo. (6), by gas-liquid chromatography according to the procedure of Zumwalt et al. (29).

CAEs and CDC type-specific antisera. CAEs and CDC type-specific antisera for quality control and identification of fractions were kindly supplied by Richard R. Facklam, Hazel Wilkinson, and William K. Harrell, CDC. R-28 antigen fractions were tested for serological activity by immunodiffusion according to the method of Ouchterlony (21).

Purified antigens. R-28 antigen was purified from group A (Lancefield strain C510) and group C (Lancefield strain B337) streptococci according to a modification of the method of Lancefield and Perlmann (14); the antigen was kindly supplied by William K. Harrell. Teichoic acid (8) was prepared according to the procedure of Moskowitz (19), from a strain of group A *Streptococcus*, also supplied by William K. Harrell. It contained E4 and polyglycerophosphate antigens identified by immunoelectrophoresis (28) against a standard purified polyglycerophosphate supplied by Maclyn McCarty of Rockefeller University. "T-28 precipitating antigen" was prepared from strain 0050 by the original method of Pakula (22), as modified by McLean (16) and Erwa (2), and was later demonstrated to form two pepsin-sensitive lines of identity with R-28 antigen prepared by either the method of Lancefield and Perlmann (14) or by HA column chromatography (Fig. 8); it therefore lacks T-28 antigen and contains R-28 antigen.

R-28, T-28, and teichoic acid antisera. Unabsorbed R-28 antiserum was prepared by repeated intravenous immunization of rabbits with heat-killed whole cells of group A R-28 streptococci (designated pool A, 7/30/71), supplied by William K. Harrell. This unabsorbed R-28 antiserum lacked bactericidal activity for homologous R-28 streptococci and cross-reacted with absorbed T-28 antiserum.

Absorbed T-28 antiserum (designated lot 1, 12/15/71) was prepared in rabbits with trypsinized, formalin-killed whole cells of group A T-28 streptococci and adsorbed with suspensions of T6 Glossy organisms according to the procedure of Moody et al. (18). It was kindly supplied by William K. Harrell. It is identical to the standard T-agglutinating antisera supplied by the CDC except that it was concentrated approximately two- to fourfold when reconstituted with distilled water. Two lines of identity were formed with unabsorbed R-28 antiserum and the absorbed T-28 antiserum when they were tested against either CAEs or HA-purified R-28 antigen.

Unabsorbed teichoic acid antiserum (designated M49, E4 pool D, 4/20/72) was prepared in rabbits against heat-killed group A, M-type 49 whole streptococci and was rich in polyglycerophosphate and E4 antibodies when tested with immunoelectrophoresis by the technique of Wilson and Wiley (28). It was also supplied by William K. Harrell.

Enzymatic degradation of R-28 antigen and of T-28 precipitating antigen. The effect of trypsin and pepsin on immunodiffusion reactivity of HA column-purified R-28 antigen and T-28 precipitating antigen was compared with that of R-28 antigen prepared from another group A *Streptococcus* (strain C510) by the method of Lancefield and Perlmann (14). The technique was that of Johnson and Vosti (9), except that the buffer system was 0.01 M instead of 0.3 M ammonium carbonate, pH 8.3; and, in the case of pepsin, equal volumes of 0.2 N HCl were added to the buffer to lower the pH for enhanced pepsin activity. Trypsin digestion was performed with trypsin containing a chymotrypsin inhibitor (9), and pepsin digestion was performed with a freshly prepared 0.25% aqueous solution of 2× crystallized pepsin (obtained from General Biochemicals, Inc., Chagrin Falls, Ohio). Digestion and control mixtures were shaken gently for 90 min at 25 C, and activity was tested by immunodiffusion after the mixtures were cooled at 4 C.

T-agglutination. Agglutination was performed according to the method of Moody et al. (18) with 0.3-ml serum samples that were serially diluted twofold with phosphate-buffered saline, pH 7.2. For agglutination inhibition, 0.1 ml of inhibitor antigen in phosphate-buffered saline (0.1 ml of phosphate-buffered saline only for controls) was mixed with 0.3 ml of each serially diluted serum; the mixtures were incubated at 37 C for one h, and held overnight at 4 C. All sera were examined for the presence or absence of precipitation and centrifuged. Supernatants of inhibited sera were then tested for agglutinin titers against a trypsinized suspension of strain 0050 (M-28), R-28 group A *Streptococcus* prepared for T-agglutination as outlined by Moody et al. (18).

Rabbit immunization. New Zealand white rabbits were given 1.0 mg (dry weight) of protein of HA column-purified R-28 antigen (0.10 M protein eluate), dissolved in 1.0 ml of sterile normal saline and emulsified with 1.0 ml of complete Freund adjuvant. Injections were given subcutaneously into the interscapular space. Six months later a booster dose of 100 μg of protein dissolved in 2.0 ml of sterile normal saline was injected intramuscularly. The animals were bled before immunization and at two-week intervals after immunization. Sera were stored frozen at −20 C without preservatives. Serum precipitins were assessed by immunodiffusion (21), and bactericidal activity was analyzed according to the method of Lancefield (11).

RESULTS

Fractionation with ammonium sulfate. The overnight growth of 120 liters of Todd-Hewitt broth yielded 126 g of whole wet strain 0050, group A (M-28) R-28 streptococci. Crude acid extraction produced 2,280 mg of protein from the 126 g for a yield of 18.1 mg of protein per g of wet streptococci. Fractionation of the 2,280 mg of CAE protein with ammonium sulfate yielded 585 mg of protein, and a final yield of 4.6 mg of ammonium sulfate protein per g of wet streptococci was obtained. Thus, only 25.7% of the CAE protein was precipitated by 60% saturation with ammonium sulfate.

Column chromatography. Further purification of R-28 antigen by HA column chromatography of the ammonium sulfate fractions resulted in 90% recovery of protein applied to the column. Approximately 4% of recovered protein appeared in the 0.01 M phosphate void volume, 44% in the 0.10 M eluate, and 52% in the 0.30 M eluate. Final yields of 0.18 mg of protein per g of wet, whole streptococci (0.01 M eluate), 1.85 mg/g (0.10 M eluate), and 2.15 mg/g (0.30 M eluate) were obtained. This represents a 10-fold soluble protein purification of 233 mg of 0.10 M HA protein eluate from 2,280 mg of CAE protein.

The pattern of elution of the R-28 antigen is shown in Fig. 1. Only the 0.10 M eluted protein (tubes no. 61 to 120) was reactive with unabsorbed R-28 antisera on immunodiffusion.

Amino acid composition. Lyophilized 1.0-mg samples of 0.10 M HA column fractions of R-28 antigen were analyzed for total quantitative amino acid composition (Table 1). HA column-purified R-28 antigen had an amino acid composition that lacked arginine, methionine, histidine, cystine, and hydroxyproline and had low amounts of tyrosine. Aspartic acid, lysine, valine, threonine, glutamic acid, and alanine were the six most predominant amino acids.

PAGE. Figure 2 presents PAGE patterns of ammonium sulfate fractions before HA column chromatography and patterns of HA column-eluted protein fractions of purified R-28 antigen obtained by the technique of Davis (1). Multiple PAGE bands were present in both ammonium sulfate and HA column-purified R-28 antigen. Figure 3 demonstrates plots of 100 log ($R_f \times 100$) versus gel percentage for 0.10 M eluted R-28 antigen and indicates that, by the criteria of Hedrick and Smith (7), HA column-purified R-28 antigen is a mixture of at least one pair of charge and three families of size isomers. Slicing and elution of the gels of 3, 6, 9, and 12%

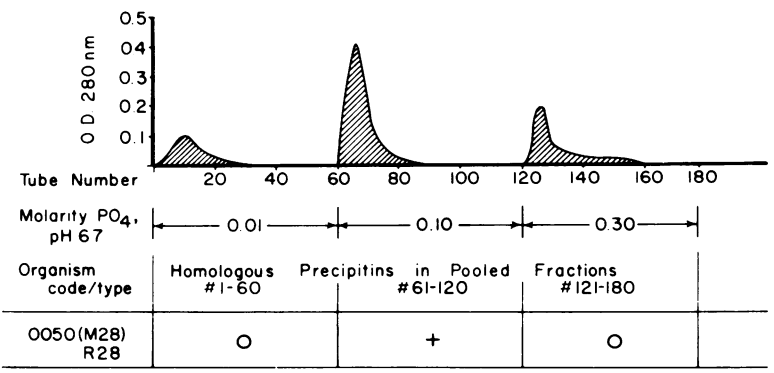

FIG. 1. *HA column elution pattern of ammonium sulfate-precipitated R-28 antigen.*

TABLE 1. *Quantitative amino acid analysis[a] of HA column-purified R-28 antigen*

Amino acid[b]	μmol/100 μmol of amino acid
Aspartic acid	15.17
Lysine	11.83
Valine	10.10
Threonine	9.78
Glutamic acid	8.94
Alanine	8.88
Glycine	8.05
Phenylalanine	7.80
Proline	7.38
Leucine	4.36
Serine	4.14
Isoleucine	2.38
Tyrosine	1.19
Arginine, Methionine, Histidine, Cystine, Hydroxyproline	0

[a] Tryptophan is destroyed by prior acid hydrolysis and is not detected in these samples. Cysteine is converted to cystine; asparagine and glutamine are converted to aspartic and glutamic acids, respectively.

[b] In addition to the amino acids reported, an unidentified peak preceding the elution of alanine was noted; this peak was not β-alanine, NH_3/NH_4+, phosphate, or $HCO_3^-/CO_3^=$

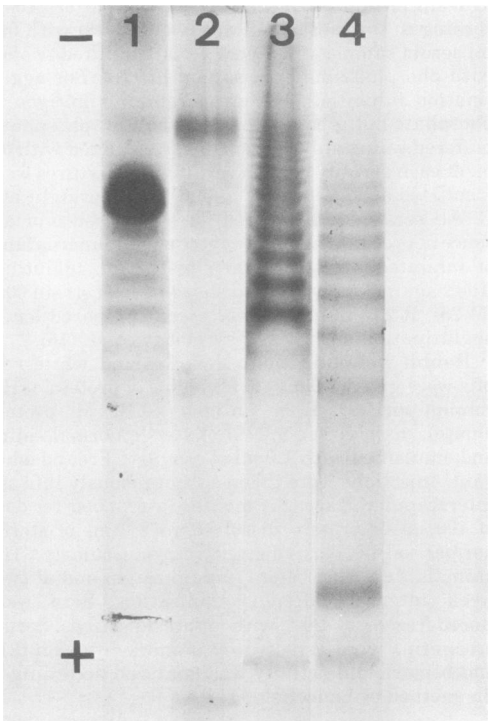

FIG. 2. *PAGE patterns of ammonium sulfate-precipitated and HA column-purified R-28 antigen. Migration is from top to bottom, towards the anode, according to the method of Davis (1). Gels: 1, Ammonium sulfate protein applied to HA column, 500 μg; 2, 0.30 M HA column protein eluate, immunodiffusion unreactive, 500 μg; 3, 0.10 M HA column protein eluate, immunodiffusion reactive R-28 antigen, 600 μg; 4, 0.01 M HA column protein eluate, immunodiffusion unreactive, 600 μg.*

acrylamide reveal that both predominant and faint bands are serologically active.

Serological and enzymatic identification of M-, T-, and R-antigens, teichoic acid, E4 antigen, and group A carbohydrate. Two precipitin lines of identity developed when CAEs and HA column-purified R-28 antigen were reacted with unabsorbed R-28 antiserum, absorbed T-28 antiserum, and rabbit serum 10 weeks postimmunization (Fig. 4). Immunized rabbits did not develop bactericidal antibody. The CAE reacted but HA column-purified R-28 antigen did not react with group A antiserum.

Figure 5 demonstrates two precipitin lines in the R-28/anti R-28 systems when HA-purified R-28 antigen was used. However, when R-28 antigen from either group A or group C streptococci was purified by a modification of the method of Lancefield and Perlmann (14), only a single precipitin line formed in the R-28/anti R-28 system and gave a reaction of identity with one of the precipitin lines formed by the HA-purified R-28 antigen. Absorbed T-28 antiserum also developed the same two precipitin lines (of identity) formed by unabsorbed R-28 antiserum when tested against HA-purified R-28 antigen.

Figure 6 reveals that neither CAEs nor HA column-purified R-28 antigen from strain 0050 contained detectable streptococcal teichoic acid or E4 antigen, whereas R-28 antigen purified from group C streptococci by the method of Lancefield and Perlmann (14) contained trace amounts of streptococcal teichoic acid and/or E4 antigen when compared with streptococcal teichoic acid prepared by the method of Moskowitz (19).

Figure 7 demonstrates that pepsin destroyed all reactivity of both ammonium sulfate precipitated and HA column-purified R-28 antigen. Ammonium sulfate-purified R-28 formed a third immunodiffusion line in comparison to the two formed by HA-purified R-28. Trypsin released a new antigen from ammonium sulfate-precipitated R-28 antigen, which formed a line of partial identity with a line seen in ammonium sulfate-precipitated R-28 antigen, and a line of complete identity with a line seen in trypsin-treated HA columns purified by R-28 antigen.

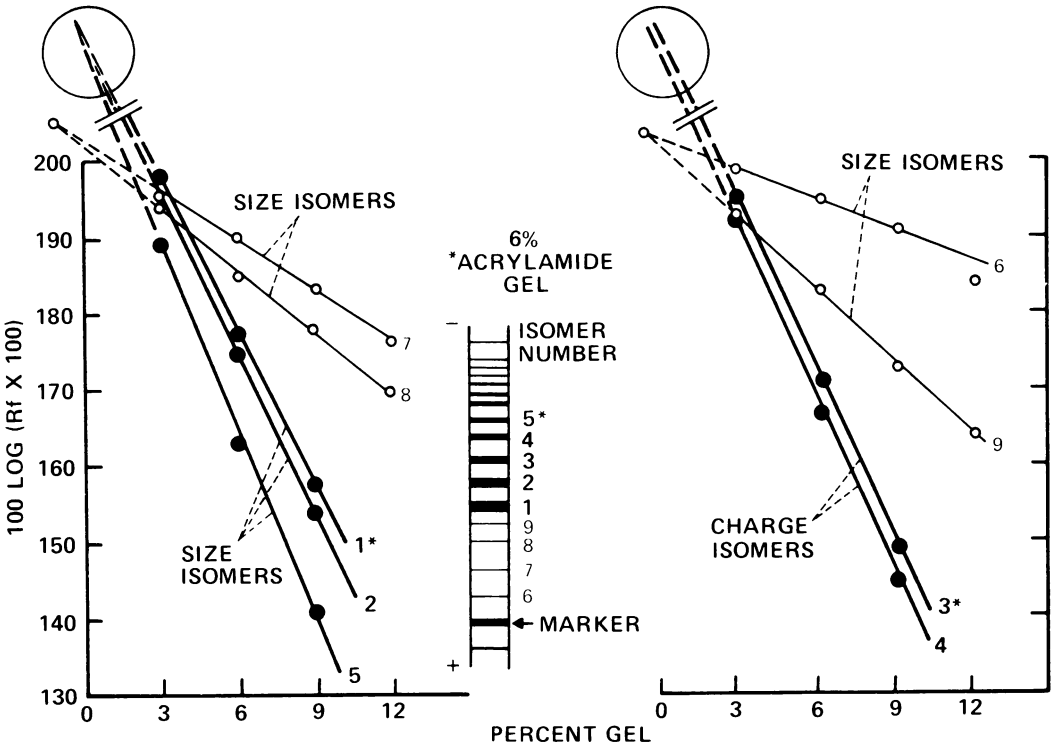

FIG. 3. *Hedrick and Smith (7) plots of 100 log ($R_f \times 100$) versus percentage of acrylamide gel for five predominant and four faint PAGE bands of HA-purified R-28 antigen. Size isomers (slopes different, lines nonparallel): 1 family, isomers 1, 2, and 5; 2 pairs, isomers 6 and 9, 7 and 8. Charge isomers (slopes similar, lines parallel): 1 pair, isomers 3 and 4.*

Figure 8 demonstrates that pepsin destroys reactivity of R-28 antigen purified from group A strain C510, and of HA column-purified R-28 antigen and T-28 precipitating antigen, both of which are prepared from group A strain 0050. Thus, T-28 precipitating antigen prepared from strain 0050 appears to be identical to R-28 antigen.

T-agglutination results (Table 2) indicate that HA-purified R-28 antigen is the best inhibitor against either unabsorbed R-28 antiserum or serum from rabbits immunized with HA-purified R-28 antigen. HA-purified R-28 antigen, R-28 antigen purified from group A streptococci by a modification of the method of Lancefield and Perlmann (14), and T-28 precipitating antigen (which contains R-28 antigen) equivalently inhibited agglutination with absorbed T-28 antiserum.

DISCUSSION

Because of previous experience with the successful purification of streptococcal M-proteins by HA column chromatography (9, 24), the same methods were applied in this study for the purification of R-28 antigen using an M-protein-poor, R-antigen-rich strain instead of an M-protein-rich strain of group A *Streptococcus*. This organism was subsequently found to lack extractable T-28 antigen and M-protein on immunodiffusion.

R-28 antigen was isolated from group A strain 0050 (M-28) R-28 *Streptococcus* in yields comparable to that achieved in isolating M-protein from many group A streptococcal M-types. The HA column elution pattern of purified R-28 antigen differed from that for type 12 (9) and other types of M-protein (Kenneth L. Vosti, personal communication; unpublished data) only in that M-proteins are usually reactive in 0.30 M HA eluates and only occasionally in 0.10 M eluates. The quantitative amino acid composition of HA-purified R-28 antigen resembled that described previously for many types of streptococcal M-proteins (3, 5, 20, 23, 24) but differed in that R-28 antigen contained relatively more valine, phenylalanine, and threonine and less alanine and glycine than types 12, 13, and 24 M-protein (unpublished data). PAGE patterns of HA column-purified R-28 antigen

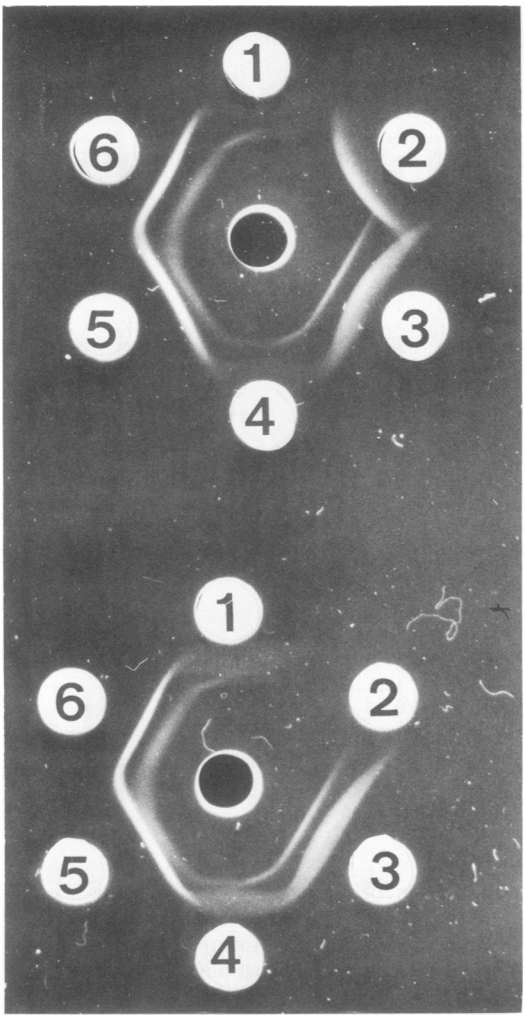

FIG. 4. *Immunodiffusion patterns of R-28 antigen with R-28, T-28, and group A Streptococcus antisera and with sera of immunized rabbits. (Upper): Central well, 0050 (M-28) R-28 CAE; wells 1 and 4, serum from rabbit 47, 10 weeks postimmunization; well 2, standard CDC group A antiserum; wells 3 and 6, unabsorbed R-28 antiserum; well 5, absorbed T-28 antiserum. (Lower): Central well, 0050 (M-28) R-28 0.10 M HA eluate; wells 1 and 4, serum from rabbit 47, 10 weeks postimmunization; well 2, standard CDC group A antiserum; wells 3 and 6, unabsorbed R-28 antiserum; well 5, absorbed T-28 antiserum.*

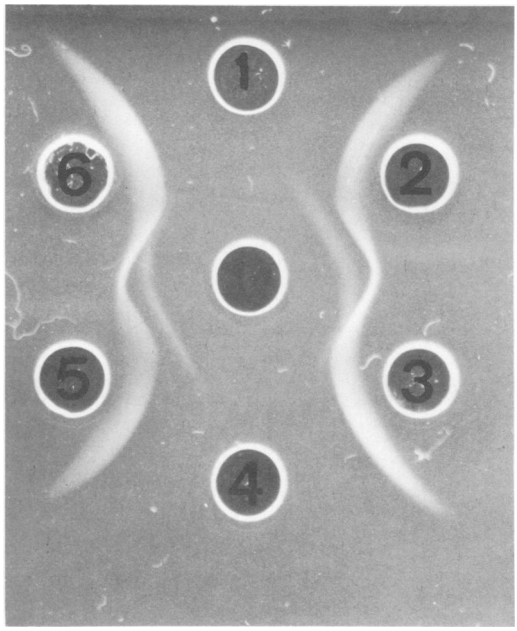

FIG. 5. *Immunodiffusion patterns of HA column-purified R-28 antigen with R-28 antigen prepared from another group A and a group C Streptococcus by trypsinization of whole cells (method modified from Lancefield and Perlmann [14]). Central well, 0050 (M-28) R-28 0.10 M HA eluate; well 1, R-28 antigen prepared from group A strain C510 (14); wells 2 and 5, unabsorbed R-28 antiserum; wells 3 and 6, absorbed T-28 antiserum; well 4, R-28 antigen prepared from group C strain B337 (14).*

were also similar to those described for different types of M-proteins (3–5, 9, 20, 23). This study demonstrates in HA-purified R-28 antigen the existence of multiple serologically active charge and size isomers by the criteria of Hedrick and Smith (7); a multiple molecular subunit structure has also been claimed for M-protein (3, 4).

These results demonstrate that HA column-purified R-28 antigen and the T-28 precipitating antigen are identical in strain 0050. In contrast to other group A streptococci, where T-antigens have been implicated in agglutination reactions (10, 13, 18), in group A strain 0050 R-28 antigen was responsible for agglutination and T-antigen could not be demonstrated. Both HA-purified R-28 and T-28 precipitating antigen have a common R antigen demonstrated to be the same R-28 antigen purified by a different method by Lancefield and Perlmann (14). In addition, a pepsin-sensitive antigen was identified that was not group A carbohydrate, M-protein, T-antigen, polyglycerophosphate, E4 antigen, or M-associated protein. By enzymatic criteria, both this antigen and the third antigen present in ammonium sulfate fractions were R-antigens or R-like antigens. R-28 antigen purified by the method of Lancefield and Perlmann (14) lacked these other R-antigens, or R-like

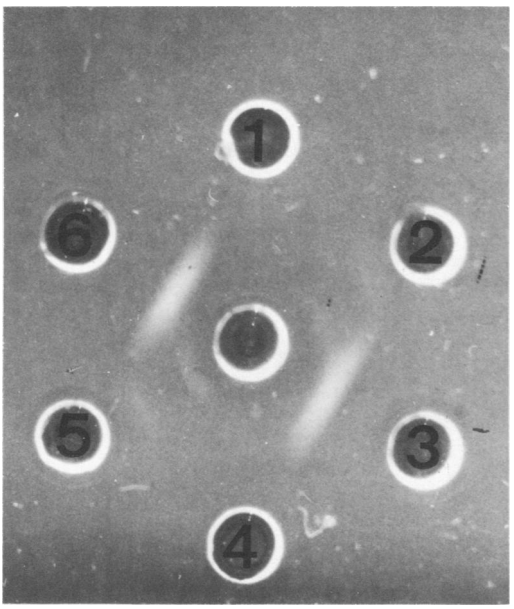

FIG. 6. *Immunodiffusion patterns of R-28 antigen with streptococcal teichoic acid (TA) (prepared by the method of Moskowitz [19]) and TA antisera. Central well, unabsorbed TA antiserum; well 1, 0050 (M-28) R-28 0.10 M HA eluate; wells 2 and 5, R-28 antigen from group C strain B337 (prepared by the method of Lancefield and Perlmann [14]); wells 3 and 6, group A streptococcal TA (19); well 4, 0050 (M-28) R-28 CAE.*

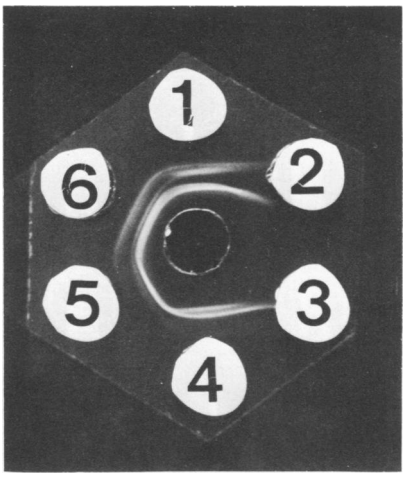

FIG. 7. *Effect of pepsin and trypsin on immunodiffusion reactivity of group A strain 0050 streptococcal R-28 antigen. Central well, unabsorbed R-28 antiserum; well 1, R-28 ammonium sulfate fraction, no enzyme; well 2, R-28 ammonium sulfate fraction, pepsin treated; well 3, R-28 0.10 M HA eluate, pepsin treated; well 4, R-28 0.10 M HA eluate, no enzyme; well 5, R-28 0.10 M HA eluate, trypsin treated; well 6, R-28 ammonium sulfate fraction, trypsin treated.*

antigens, but contained detectable amounts of teichoic acid not demonstrable in HA column-purified R-28 antigen. Furthermore, HA column-purified R-28 antigen produced precipitin but not bactericidal activity in rabbits, as was also true for the R-28 antigens prepared from both group A and group C streptococci by the method of Lancefield and Perlmann (14). Failure to elicit bactericidal antibodies in rabbits (11) and resistance to trypsin digestion (10) are evidence that M-28 protein is not the unidentified antigen(s). Resistance to trypsin differentiates the unidentified antigen(s) from M-associated protein which is trypsin-sensitive (25). Additionally, sensitivity of this antigen to pepsin digestion separates it from T-antigens that Lancefield and Dole (13) have shown to be pepsin-resistant and trypsin-resistant. It is important to note that Lancefield (11) was unable to demonstrate the presence of T-antigen in type 28 streptococci.

It seems likely that this undefined antigen is another R- or R-like antigen since it is both pepsin sensitive and trypsin resistant (14). The demonstration of multiple R- or R-like anti-

TABLE 2. *Agglutination and agglutination inhibition titers of antisera used to agglutinate strain 0050 (M-28) R-28 group A Streptococcus*

Antiserum	Agglutination titer
Unabsorbed R-28 Antiserum	1:128
Inhibitor:[a]	
HA-purified R-28 antigen	1:8
R-28 antigen prepared from group A strain C510[b]	1:16
T-28 precipitating antigen	1:32
Serum from rabbit 47, 10 weeks post-immunization	1:8
Inhibitor:	
HA-purified R-28 antigen	Undiluted
R-28 antigen prepared from group A strain C510	1:4
T-28 precipitating antigen	1:4
Absorbed T-28 antiserum	1:16
Inhibitor:	
HA-purified R-28 antigen	1:4
R-28 antigen prepared from group A strain C510	1:4
T-28 precipitating antigen	1:4

[a] All inhibitor antigens, approximately 1 mg of protein per ml.

[b] Method modified from Lancefield and Perlmann (14).

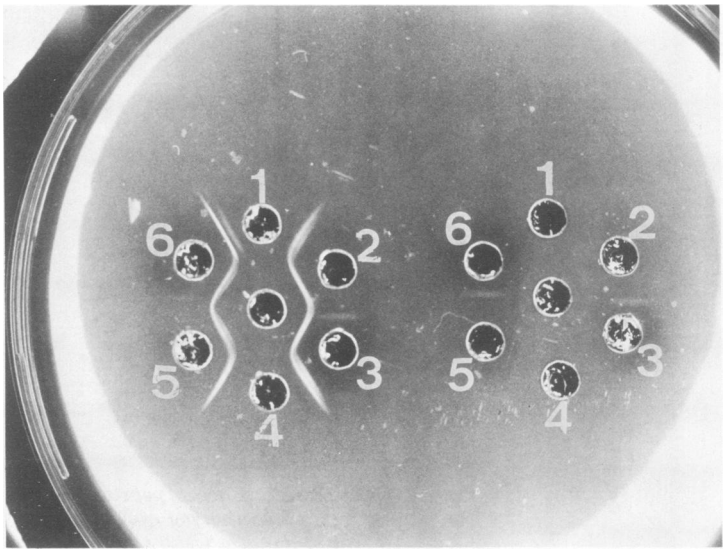

FIG. 8. *Effect of pepsin on immunodiffusion reactivity of R-28 antigen and T-28 precipitating antigen. (Left) No pepsin: central well, T-28 precipitating antigen (prepared from strain 0050 by method of Pakula [22] as modified by McLean [16] and Erwa [2]); well 1, R-28 0.10 M HA eluate; wells 2 and 5, absorbed T-28 antiserum; wells 3 and 6, unabsorbed R-28 antiserum; well 4, R-28 antigen prepared from group A strain C510 (method modified from Lancefield and Perlmann [14]). (Right) Pepsin treated: central well, T-28 precipitating antigen (same method as central well above), pepsin treated; well 1, R-28 0.10 M HA eluate, pepsin treated; wells 2 and 5, absorbed T-28 antiserum; wells 3 and 6, unabsorbed R-28 antiserum; well 4 R-28 antigen prepared from group A strain C510 (same method as well 4 above), pepsin treated.*

gens, initially shown by Wilkinson (27), indicates the diversity of these related or similar antigens. The two immunodiffusion lines seen in HA column-purified R-28 antigen may represent the two proteins noted on electrophoresis of the R-28 antigen of Lancefield and Perlmann (14), whereas the three lines seen in ammonium sulfate fractions of R-28 may reflect the presence in strain 0050 of the three R-28 antigens noted by Wilkinson (27) in her strain T28/150A/2, which was derived from strain 0050 by animal passage. The multiplicity of R-antigens demonstrable by immunodiffusion or electrophoretic criteria may be related to the physicochemically rather drastic purification techniques employed (e.g., hot acid extraction, trypsinization, ammonium sulfate precipitation, etc.). Such harsh procedures may either dissociate naturally occurring charge and size subunit isomers of R-antigens or possibly release artifacts of hydrolysis, salting-out, etc.

Additional studies are needed to further assess the relationship of the structural features of M-protein, R-antigen and T-antigen of the group A *Streptococcus* to their biological functions. That is, it remains to be determined whether differences in primary, secondary, tertiary, or quaternary structure of these topographically related surface antigens are involved in their functional differences. Results of this study suggest that M-proteins and R-antigens are quite similar subunit isomer proteins on a broad physicochemical basis and may differ only by subtle, as yet physicochemically undefined, characteristics which are currently distinguishable only by immunological, biological, and enzymatic techniques. The same may be the case for T-antigen.

Clearly, gentler physicochemical methods for the isolation and purification of M-proteins and R- and T-antigens are required to elucidate the physicochemical basis for the subtle immunological, biological, and enzymatic differences between these antigens in their native state on the group A streptococcal cell wall or surface.

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